Usage of Nonporous Polymeric Particles for Reversed-Phase High-Performance Liquid Chromatography of Oxidized and Deamidated Forms of Recombinant Human Growth Hormone

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Abstract

In this work, a C_{18} reversed-phase column with nonporous polymeric 2.5-µm particles is utilized to initially test the analysis of oxidized and deamidated human growth hormone (hGH). Phosphate buffer (pH 7.5) with 24% 1-propanol was used for elution. This quick method (analysis time is 20 min) gave a selectivity, as judged by the number of detected peaks, and resolution of hGH variants that is better than many methods in which porous silica particle columns are used. Only mixtures of oxidized and deamidated hGH are analyzed, and no characterization of the peaks is performed. The results indicate that C_{18} nonporous polymeric column material is a promising alternative for the chromatographic separation of several hGH variants.

Introduction

Human growth hormone (hGH) consists of a single polypeptide chain containing 191 amino acid residues and two disulfide bridges (1). Modifications of recombinant hGH can arise during fermentation and purification processes, as well as during storage. These modified forms must be determined, which is often performed by reversed-phase (RP) high-performance liquid chromatography (HPLC). Three methionine residues are included in the polypeptide chain; Met14 and Met125 have been reported to be susceptible to oxidation (2–4), whereas the remaining methionine (Met170) is not oxidized in the native peptide (4). Becker et al. (2) reported a greater susceptibility for oxidation of Met14 relative to Met125 in hGH. Other modified forms of hGH include dimers (5), deamidated variants of hGH (Asn149 → Asp149 and Asn152 → Asp152) (2,4), *N*-terminal truncated des-Phe (4), cleaved variants (Thr142-Tyr143) (4), and a trisulfide (Cys182-SSS-Cys189) variant (6).

RP-HPLC, when used to analyze various hGH forms, is usually performed using isocratic elution at neutral pH, with 1-propanol as the organic modifier, at a temperature of approximately 40–50ºC (3,5,7–9). Porous silica particles have been used as the preferred material for separation of hGH variants by RP-HPLC; however, porous column material suffers from giving considerable peak broadening when analyzing high-molecular-weight compounds because of the slow diffusion rate in the pores. Nonporous particles have been shown to give faster mass transfer kinetics, thereby producing peaks that are more narrow, giving higher efficiency and resolution (10). These columns also allow for a much shorter analysis time compared with columns with porous material, and, for example, the analysis of tryptic-digested proteins has been performed in less than 5 min (11).

The present study describes a method in which a column with nonporous 2.5-µm polymeric C_{18} particles was used to initially test the separation of oxidized and deamidated hGH variants by RP-HPLC. Selectivity and resolution are improved by this quick method when compared with many other methods in which porous silica material is used.

Experimental

A laboratory sample of recombinant hGH was generously provided from Pfizer (Stockholm, Sweden). Analytical-grade 1-propanol was from Labscan (Dublin, Ireland), hydrogen peroxide (suprapur) was from Kebo Lab (Spånga, Sweden), and catalase was obtained from Sigma (Munich, Germany). Sodium phosphate and ammonium bicarbonate (both analytical grade) were obtained from Merck (Darmstadt, Germany).

The oxidation was performed by incubating hGH in 300 mmol/L hydrogen peroxide in 100 mmol/L ammonium bicarbonate for 5 h at 0°C, followed by addition of catalase (ratio of catalase–hGH was 1:100, w/w) and incubation for 5 min at room temperature, to quench the reaction, as previously described (4,8). The deamidation was performed by incubating hGH (1 mg/mL) in 30 mmol/L ammonium bicarbonate for 24 h at 37°C (8).

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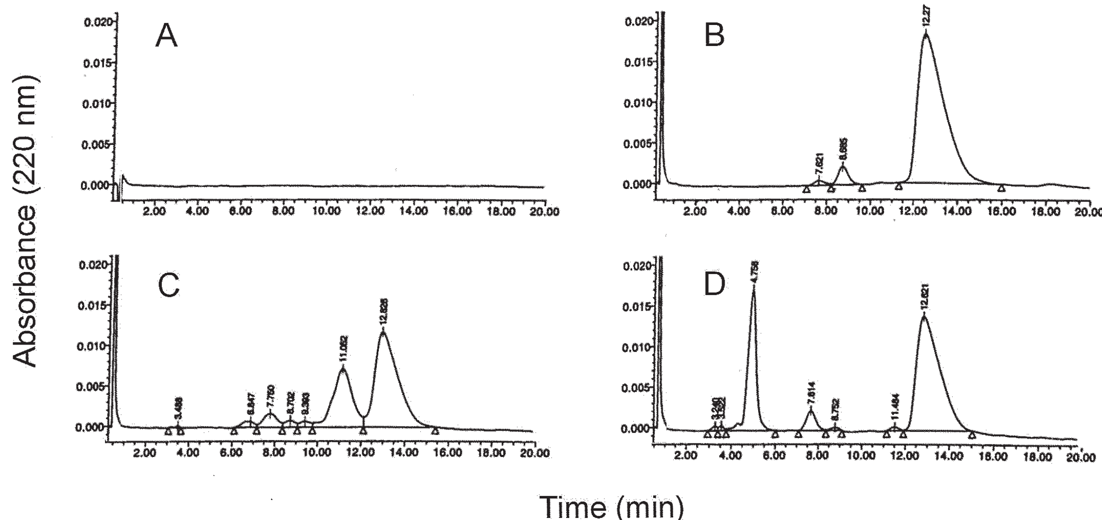

Figure 1. RP isocratic HPLC of recombinant hGH using a nonporous TSK-gel Octadecyl-NPR polymeric column equilibrated with 24% (v/v) 1-propanol in 20 mmol/L sodium phosphate (pH 7.5) at a flow rate of 1.0 mL/min and temperature 45°C. Two microliters hGH (1.3 mg/mL) was injected, and detection was performed by measurement of absorbance at 220 nm: (A) water, (B) untreated hGH, (C) deamidated hGH, and (D) oxidized hGH mixed with untreated hGH in a ratio of 1:2. Further details are described in the Experimental section.

A Waters Alliance 2695 HPLC system, with a 996 PDA UV detector and Millennium as the controlling software was used (Waters, Milford, MA). The HPLC system included a TSK-gel Octadecyl-NPR column (2.5-µm nonporous particles, 35- × 4.6-mm i.d.) (Tosohaas, Stuttgart, Germany). The column was run at 45ºC, and the samples were kept in the autosampler at 8ºC during the analysis. The mobile phase used for elution contained 24% (v/v) 1-propanol in 20 mmol/L sodium phosphate (pH 7.5). The flow rate was 1.0 mL/min, and 2 µL hGH (1.3 mg/mL) was injected. Detection was carried out by measuring UV absorbance at 220 nm.

Results and Discussion

The presented RP chromatography method, using a column with nonporous particles, allowed for a high degree of selectivity (as judged by the number of detected peaks) in the analysis of the oxidized and deamidated hGH samples (Figure 1). The untreated hGH sample gave a distinctive main peak at approximately 12.5 min, comprising 94% of the total integrated area. The oxidation and deamidation of samples was performed in exactly the same way as in previous work in which an RP-HPLC column with porous silica C_{18} material was used for separation of characterized oxidized and deamidated hGH forms (8). Therefore, this earlier work (8) was used as a comparison method when interpreting the results from the new method, considering the number of peaks, order of their elution, and approximate relative peak areas of these peaks.

The oxidized hGH sample gave a total of at least five distinct peaks, and the resolution between the two dominate peaks at 4.8 and 7.6 min and between the 7.6 min peak and the native hGH peak (at ~ 12.5 min) (or both) was better than for many other methods (3,4,7–9) in which porous silica is used. These two generated peaks in the oxidized sample (at 4.8 and 7.6 min) are believed to contain dioxidized Met14/Met125 hGH and mono-oxidized Met125 hGH, respectively, on the basis of their order of elution and relative peak area, when compared with the RP-HPLC results from an identical treated sample (8). The deamidated hGH sample gave a total of six peaks, and the deamidated main peak (probably deamidated Asn149 hGH), eluting at 11 min, just before the native hGH peak, gave an area of 30%, which is close to the value previously obtained for an identically treated sample (8). The number of obtained peaks for the oxidized and the deamidated samples (Figure 1) was slightly higher than that obtained with methods using porous particles (3–5,7–9). Also, the overall resolution between many of the peaks, in all of the hGH samples analyzed, seems to be improved when compared with other methods using porous silica. A polymeric column material, compatible with pH 2–12, was chosen because RP-HPLC of hGH forms has been most successful when used in a pH range of 7–8 (3,5,7–9), which has a degrading effect on silica.

The oxidation of hGH, using high concentrations of hydrogen peroxide, which was also used in the present study, has been reported to give a higher degree of oxidation of Met125 than Met14, even though, in the native hGH, Met14 is the primary site of oxidation (2,3). The amino acids aspargine and glutamine are sensitive to degradation, becoming deamidated to aspartic and glutamic acid, respectively (isoaspartic acid may also be formed). Becker et al. showed that Asn149 is the amino acid in hGH that is most susceptible to deamidation (2). Deamidation reactions have been shown to be influenced by pH, temperature, and ionic strength, as well as by the adjacent amino acid sequence. In the present study, increased temperature and alkaline pH were used to induce deamidation.

Nonporous particles have the advantage of giving reduced peak broadening and higher efficiency and resolution when compared with porous particles, and columns with nonporous particles have a reduced analysis time (10). Disadvantages of the use of nonporous particles include a reduced sample loading capacity and an increased back pressure, which limits the column length and, thereby, the efficiency of the column.

Compared with several porous silica HPLC methods (3–5,7–9), the method presented here gives improved selectivity and resolution for several peaks in the oxidized and the deamidated hGH samples. The analysis time (20 min) is also short compared with many other RP-HPLC methods in which 30–60 min/injection is required in the analysis of hGH (4–9). No characterization of the eluted peaks was performed, and purified forms were not used for injection. Thus this characterization, and possibly a further optimization of the method, remains to be performed. This work was mainly performed as an initial test, using nonporous column material to analyze oxidized and deamidated hGH forms.

In conclusion, the results from the presented RP-HPLC method indicate that C_{18} nonporous polymeric column material is a promising alternative for the separation of several hGH variants.

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